Iron requirement and chelator production of staphylococci, *Streptococcus faecalis* and enterobacteriaceae

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The effect of iron deprivation on growth of 101 aerobic strains of gram-positive and gram-negative bacteria was studied on agar media in the presence of various concentrations of the synthetic iron chelator ethylene diamine di-orthohydroxyphenyl acetic acid (EDDA) and the iron binding protein transferrin.

Growth of *Staphylococcus epidermidis* was inhibited by 15 mM EDDA and 1.5 mM transferrin. *Staphylococcus aureus* was only inhibited by 44 mM EDDA and not by transferrin. None of the strains of *S. faecalis* was inhibited. The majority of the enterobacteriaceae (*E. coli*, *Salmonella* spp, *Klebsiella* spp) was inhibited by 44 mM EDDA and 1.5 mM transferrin. The relation between susceptibility and concentration of EDDA and transferrin was expressed as S-value for each species. Iron supply with various iron compounds could restore the effects of inhibition.

In all species except in *S. faecalis* iron chelator production could be demonstrated, using indicator plates of media containing EDDA and flooded with 10^4–10^5 colony forming units of indicator organisms.

The iron chelator of both *S. epidermidis* and *S. aureus* could stimulate growth of *S. epidermidis*, but not that of enterobacteriaceae. Iron chelators from all gram-negative bacteria were functionally interchangeable, but did not stimulate growth of gram-positive bacteria.

INTRODUCTION

Iron is required by many bacteria as an essential constituent of metabolic enzymes, as catalase, peroxydase and cytochromes. Limitation of iron will result in a reduction of metabolic activity and growth. The level of free ionic iron in body fluids containing iron-binding proteins like transferrin or lactoferrin is too low for bacterial growth and can be considered as a defence mechanism. In *in-vivo* experi-

ments an inhibitory effect of bacterial growth by lactoferrin and transferrin could be demonstrated in *Escherichia coli* (Bullen and Rogers, 1969; Bullen, Rogers and Leigh, 1972; Masson and Heremans, 1971; Rogers, 1973), *Clostridium welchii* (Rogers, Bullen and Cushnie, 1970), *Shigella dysentariae* (Schade and Caroline, 1944; 1946), mycobacteria (Kochan, Pellis and Golden, 1971) and staphylococci (Schade, 1963). By saturating the iron-binding proteins with iron or adding iron compounds this inhibitory effect could be abolished (Bullen, Cushnie and Rogers, 1967; Schade, 1963).

For the uptake of environmental iron bacteria synthesize a number of iron-chelating substances as shown in some strains of *E. coli* (Brot and Goodwin, 1968; Rogers, 1973; Kochan, Kvach and Wiles, 1977), *Aerobacter aerogenes* (O'Brien and Gibson, 1970), *Salmonella typhimurium* (Pollack and Neilands, 1970), mycobacteria (Snow, 1965) and bacilli (Garibaldi and Neilands, 1956). These chelators include the secondary hydroxamates, like mycobactins, desferrioxamine, schizokinen and the phenolates like enterochelin, the cyclic trimer of 2,3-dihydroxybenzoylserine. Probably these bacterial iron chelators can compete with transferrin and lactoferrin as shown by Schade (1963) in *S. aureus* in normal rabbit serum. Rogers (1973) showed a direct relationship between the ability to synthesize these substances and virulence of *E. coli* for mice. Miles and Khimji (1975) studied inhibition of bacterial growth by the synthetic iron chelator ethylene diamine di-ortohydroxyphenyl acetic acid (EDDA) of 61 strains within 6 species belonging to the enterobacteriaceae and pseudomonadales on solid media and demonstrated the production and the interchangeability of bacterial iron chelators.

Less is known about the iron chelators of other bacteria and the mechanism of competition with iron-binding proteins.

In this study growth of a series of aerobic gram-negative and gram-positive bacteria is tested in the presence of various concentrations of EDDA and transferrin. For this purpose the methods of Miles and Khimji (1975) were modified to increase the sensitivity of the test.

The effect of iron supply and the production of bacterial iron chelators in all strains were studied. Finally the interchangeability of these chelators between all species was tested.

MATERIALS AND METHODS

Bacteria. The strains used are given in Table 1.

Chemicals. Ethylene diamine di-orthohydroxyphenyl acetic acid (EDDA, K and K laboratories), freed from contaminations by the method of Rogers (1973). Stock-solutions (20 mg/ml) were made by dissolving EDDA in 1 N NaOH (56C) and adjusting the pH to 7.0 by 10 N HCl. Chelex 100 (Biorad Richmond C.A.) was used batchwise, to reduce the iron content of our media as described by Willard,

Table 1. Bacterial strains used in this study; in parenthesis the number of strains tested

Bacteria	Species and strains
Salmonella	*S. typhimurium* NCTC 5094. *S. typhimurium* (9), *S. newport*, *S. dublin*, *S. kentucky*, *S. chester*, *S. brandenburg* (2).[1]
Escherichia coli	NCTC 2276, 9001, ATCC 4157. Enteropathogenic strains: 055, 011, 020, 026, 086, 0119, 0124, 01259 and 0126.[2] Non-enteropathogenic strains: D1, D2, 12 BA and 12 C.[1]
Klebsiella	*K. pneumoniae* (16).[1]
Proteus	*P. vulgaris* (6).[1]
Pseudomonas	*Ps. aeruginosa* (10).[1]
Streptococcus	*S. faecalis* (18).[3]
Staphylococcus	*S. aureus*. NCTC 9315, 8358, 10033, 8717, 8319, 10039, 10457, 9754, 8329, 8509, 8510, 8511, 8354, 8356, 30435, 8325.
	S. epidermidis (19).[4]

[1] Obtained from human infections, collected by the department of Clinical Bacteriology of the Laboratory of Microbiology, Utrecht, (L.v.M.).
[2] Obtained from the National Institute for Public Health (R.I.V.), Bilthoven.
[3] Obtained from Dr. J. A. A. Hoogkamp-Korstanje (L.v.M., Utrecht).
[4] Obtained from Dr. J. Verhoef (L.v.M., Utrecht).

Davis and Wood (1969). Apo-transferrin (Behring-Werke A.G., Marbourg Latin), containing less than 20 μg Fe/g.

Media. Nutrient Broth (Oxoid no. 2) supplemented with 0.25% glucose was used as basal medium for all strains except *S. faecalis*. For *S. faecalis* the glucose concentration was 1%. The iron content of this medium was 0.48 μg/ml and after Chelex 100 treatment 0.19 μg/ml. For pour-plates the basal medium was supplemented with 1.7% agarose (L'Industrie Biologique Francaise, Gennevilliers). There was no increase of the iron content of the medium with added agarose.

Assay of iron. The iron content of the media was measured by A.A.S. on a Perkin and Elmer Atomic Adsorption Spectrofotometer (flameless) type 300 S.G. and with the Tripyridyl method as described by Mehlig and Shepherd (1947).

EDDA and transferrin susceptibility of bacteria. 5 ml of basal agar-medium was poured into glass petridishes with 5 cm diameter, resulting in an agarlayer of 3 mm thickness. Cups of 2.5 mm diameter were cut out in the agar. The plates were flooded with 3 ml of a 10000 fold diluted 18 h culture of bacteria in basal medium. After removal of the spare fluid, especially out of the cups, the surface of the agar-plates contained 10^4–10^5 bacteria just not sufficient to produce confluent growth.

After drying the plates (1 h at 45 C) 5 μl of solutions containing EDDA (1.5; 4.4;

15 and 44 mM) or transferrin (0.2; 0.5 and 1.5 mM) were pipetted into the cups. The highest concentrations are near the maximal solubility of EDDA and transferrin at pH 7.

The diameters of the zones of inhibition, appearing after 18 h at 37 C were measured. To compare the susceptibility to EDDA and transferrin the S(usceptibility)-value was introduced according to the theory of diffusion in agar media (Cooper, 1955):

$S = X^2/{}^e\log c$ X is the diameter of zones of inhibition (mm).
c is the concentration of EDDA or transferrin (mM).

Reconstitution. Iron was supplied by adding little iron rings of 2 mm diameter or iron salts (iron dicitrate, iron ammonium citrate, iron nitrilotriacetate) in various concentrations to the cups of the plates.

Detection of iron chelators by indicator plates. To detect iron chelators 0.1–0.5 ml of EDDA solutions, ranging from 5–50 mM were mixed with 5 ml molten basal agar and poured into petridishes of 5 cm diameter. After cooling the plates were flooded with 10^4–10^5 colony forming units (c.f.u.) of indicator strains. As indicator strains were used: *S. epidermidis* S 87, *K. pneumoniae* 109, *P. vulgaris* D1 and *E. coli* 12 BA. These strains appeared to be the most susceptible to EDDA and were inhibited by the concentration of EDDA used. 101 strains belonging to 6 species were tested for chelator production by making spot inocula of 10^7 c.f.u. (too dense for inhibition by EDDA in the concentrations used in the indicator plates) from 18 h cultures in basal medium on the indicator plates. After 24 h at 37 C, production of chelators by the tested strains during growth will result in growth of the indicator strains, if functional for the latter. The diameter of the stimulation zones were estimated from + to + + +.

Statistical analysis. The range and median of the S-values were determined for each species. The Wilcoxon test and the chi-square test were performed to analyse the statistical significance of the results. p-Values over 0.01 are considered to be non-significant (NS).

RESULTS

Inhibition of growth by EDDA and transferrin. Fig. 1 shows the zones of growth inhibition of *S. epidermidis* after 18 hours at 37 C in the presence of various concentrations of EDDA. Similar results were obtained with a number of strains (45) of *S. aureus*, *E. coli*, *Salmonella* spp., *Klebsiella* spp., *Proteus* spp. and *Ps. aeruginosa*. The zones of inhibition varied with and within the species tested. Plots of the diameters of inhibition zones were approximately linear with respect to $^{10}\log$ concentrations of EDDA. This is shown in fig. 2 for a representative susceptible strain of each species.

The variation of susceptibility within the enterobacteriaceae and pseudo-

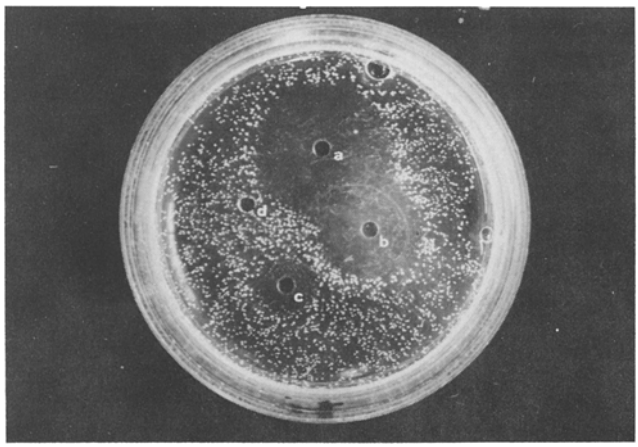

Fig. 1. Inhibition of growth of *S. epidermidis* after 18 h at 37 C by EDDA in various concentrations. a: 15 mM EDDA. b: 4.4 mM EDDA. c: 1.5 mM EDDA d: 0.4 mM EDDA.

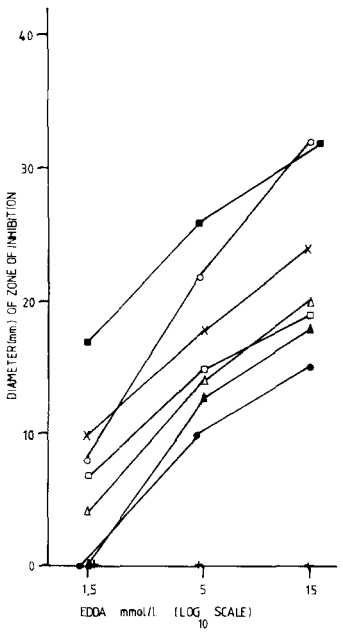

Fig. 2. Relationship between EDDA concentrations and zones of inhibition in 8 representative strains.
○: *S. epidermidis* S 87, ●: *S. aureus* NCTC 8319, △: *E. coli* 12 C, ▲: *S. typhimurium* D 13, □: *K. pneumoniae* D 5, ■: *P. vulgaris* D 4, × : *Ps. aeruginosa* Ch 1, + : *S. faecalis* N 31.

Table 2. Ranges and medians of S-values of EDDA and transferrin in three species of gram-positive and three species of gram-negative bacteria

Species	EDDA			Transferrin		
	Number of strains inhibited[1]	Range of S-value of susceptible strains	Median of S-value	Number of strains inhibited[1]	Range of S-value of susceptible strains	Median of S-value
S. epidermidis	19 (19)	2.2–4.2	3.3	19 (19)	2.1–3.6	2.7
S. aureus	11 (16)	0.7–3.5	1.4	0 (16)	-	-
S. faecalis	0 (18)	-	-	0 (18)	-	-
E. coli	15 (16)	1.6–2.3	2.1	12 (16)	1.4–4.5	2.1
Salmonella	10 (16)	0.4–2.5	0.8	12 (16)	1.0–2.1	1.7
Klebsiella	15 (16)	0.4–2.7	1.9	13 (16)	0.8–3.6	2.2

[1] The number of tested strains is given in parenthesis.

monadales was small, in general *Proteus* spp. were most susceptible. Within the group of gram-positive cocci strains of *S. epidermidis* were highly susceptible, strains of *S. aureus* were less and strains of *S. faecalis* were not susceptible at all.

Within 3 species of gram-positive bacteria (53 strains) and 3 species of gram-negative bacteria (48 strains) the lowest concentrations of EDDA and transferrin inhibiting growth were determined and expressed as S-values. The ranges and the medians are given in Table 2.

All strains of *S. epidermidis* (19) were inhibited by 15 mM EDDA and 1.5 mM transferrin. 11 strains of *S. aureus* (69%) were inhibited by 44 mM EDDA, 5 strains (31%) were not inhibited by the highest concentration of EDDA (44 mM) soluble in the medium. None of the strains were inhibited by transferrin. All strains of *S. faecalis* were completely insusceptible to the highest concentrations of EDDA and transferrin.

15 strains of *E. coli* (94%), 15 strains of *Klebsiella* (94%) and 10 strains of *Salmonella* (62.5%) were inhibited by 44 mM EDDA. The other strains tested were not susceptible to this concentration.

12 strains of *E. coli* (75%), 13 strains of *Klebsiella* (81%) and 12 strains of *Salmonella* (75%) were inhibited by 1.5 mM transferrin. The other strains were not inhibited.

The median of the S-value of strains of *Salmonella* susceptible to EDDA is significantly lower than that of the other gram-negative bacteria ($p < 0.001$). In species susceptible to EDDA and transferrin the S-values to both substances are comparable, except in *Salmonella* (NS). The difference in S-value of *S. aureus* and *S. epidermidis* both for EDDA and transferrin is statistically significant ($p < 0.001$). It can be said that *S. epidermidis* is more susceptible to EDDA and transferrin than *S. aureus*.

Effect of ironsupply. The assumption that growth inhibition is due to a deficiency of available iron in the medium is confirmed by the effect of iron supply. In all strains inhibition could be abolished if iron was supplied in excess, by filling the testcups in the agar with iron dicitrate, iron ammonium citrate, iron nitrilotriacetate or metallic iron.

Chelator production. Fig. 3 shows the stimulation of growth of indicator strain *S. epidermidis*, inoculated on the agarsurface, by chelator production of 4 strains of *S. epidermidis*. The same effect is achieved by the supply of iron. Table 3 shows the chelator production of 101 strains on four indicator strains. All bacteria except *S. faecalis* produced iron chelators, detectable in this system.

The iron chelator of *S. epidermidis* as well as that of *S. aureus* is used by *S. epidermidis*.

Within the enterobacteriaceae the chelators could be used by all three gram-negative indicator strains even not belonging to the same species.

There is no functional interchangeability of iron chelators between staphylococci and enterobacteriaceae.

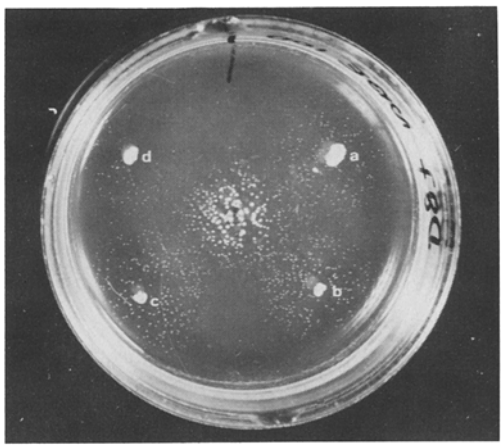

Fig. 3. Growth stimulation of indicator strain *S. epidermidis* S 87 on agar containing 0.21 μmol EDDA, by 4 strains of *S. epidermidis*. a: S 87; b: S 102; c: P_1; d: S 1520 and by metallic iron (center).

DISCUSSION

Modification of the method of Miles and Khimji (1975) using thinner agar layers and smaller cups resulted in enlarged zones of inhibition (twice as large at the same concentrations of EDDA used), and thus, in an increased sensitivity of the test. In this way we were able to demonstrate growth inhibition of *S. aureus* by EDDA in 69% of the strains, whereas this was not possible using agar plates

Table 3. Stimulation of growth of 4 indicator strains[1] on plates with EDDA by 6 species of bacteria and iron

Chelator producing strains	Indicator strains			
	S. epidermidis	*E. coli*	*K. pneumoniae*	*P. vulgaris*
S. epidermidis	++[2]	—	—	—
S. aureus	++	—	—	—
S. faecalis	—	—	—	—
E. coli	—	++	++	++
Salmonella	—	++	+	++
Klebsiella	—	++	++	+
Ring of metallic iron	+++	+++	+++	+++

[1] Indicator strains: *E. coli* 12 BA, *K. pneumonia* 109, *P. vulgaris* D 1, and *S. epidermidis* S 87.
[2] Diameter of zones of growth stimulation in mm: —no stimulation; + 10 mm; ++ 20 mm; +++ 25 mm.

according to Miles and Khimji (1975). It became also possible to demonstrate inhibition by transferrin *in-vitro*. Our results of growth inhibition by EDDA in enterobacteriaceae confirm the results of Miles and Khimji (1975). We found *Salmonella* significantly less susceptible to EDDA than *E. coli* or *Klebsiella*. This could not be demonstrated for transferrin.

In these *in-vitro* experiments on agar media most strains of enterobacteriaceae (77%) showed inhibition of growth by 1.5 mM transferrin. In studies using rabbit serum and human serum inhibition by transferrin was demonstrated in some strains of *E. coli* (Bullen and Rogers, 1969) and *Serratia marcescens* (Traub, 1977). There is evidence now that enterobacteriaceae in general in *in-vitro* experiments on agar media are also inhibited by transferrin.

The difference in susceptibility to EDDA of *S. aureus* and *S. epidermidis* can be explained by a higher iron requirement of *S. epidermidis* or by the production of more functionally iron-binding substances competitive with EDDA by *S. aureus*.

In this *in-vitro* growth of *S. aureus* appeared not to be inhibited by transferrin. This fact confirms the results of studies on growth of *S. aureus* in human serum (Schade, 1963). Apparently *S. aureus* is able to remove iron from transferrin, which might be considered as a virulence factor.

Iron supply restored growth completely for all strains, so it is clear that the observed inhibition effects were due to iron deprivation.

Iron chelator production was demonstrated in staphylococci and enterobacriaceae using a second modification of the method of Miles and Khimji (1975). Instead of mixing the indicator strain through the agar at 45 C, we flooded plates, containing EDDA, with 10^4–10^5 c.f.u. The advantage of inoculating only the surface of the agar is the simplification of the technique. Plates can be poured in large amounts, stored and used at any time, without re-melting before use. There is also the possibility to test other microorganisms not proof against 45 C.

A large amount of information is available about iron chelators of enterobacteriaceae. Also the interchangeability of bacterial iron chelators within the enterobacteriaceae, already described by Miles and Khimji (1975) could be demonstrated. The interchangeability of iron chelators within two species of staphylococci indicates similarity and possibly the same receptor sites in the bacterial cell wall.

As there was no functional interchangeability of chelators between staphylococci and enterobacteriaceae the assumption is justified that iron chelators of gram-positive bacteria differ from those of gram-negative ones. As iron chelators are also demonstrated in bacilli (Bakshi and Williams, 1969) and mycobacteria (Kochan et al, 1971) it can be said that chelator production is a characteristic of many bacteria.

Growth of *S. faecalis* was not inhibited by EDDA and transferrin in this *in-vitro* system. If *S. faecalis* requires iron, then the mechanism to acquire iron differs from that of staphyloccocci and enterobacteriaceae, as no chelator production could be demonstrated until now.

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